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INTRODUCTION: Dysregulation of MKK4 has been associated with cancer progression in a variety of disease states (1-3). While the preponderance of data implicate a role for MKK4 and its signaling pathway in disease, the mechanism(s) that regulate its expression have been incompletely studied. We sought to determine the mechanism by which MKK4 protein levels are modulated using high- and low-MKK4-expressing prostate and ovarian cancer cell lines as a model system. Using a variety of complementary approaches we showed that MKK4 protein is highly stable in all cell lines tested and displays no differential sensitivity to protease inhibitors (4). The compartmentalization of the MKK4 mRNA is also unchanged when comparing high- and low-expressing cells, which all show a highly stable transcript with a predominantly nuclear localization. However, within the cytoplasmic MKK4 mRNA subset, there is a distinct increase in the association of the MKK4 mRNA with the translational machinery in high-MKK4-expressing cells compared to low-MKK4-expressing cells (4). Together, these observations suggested that MKK4 protein levels could be subject to translational regulation. This mechanism of control endows cells with the ability to rapidly fine-tune levels of specific proteins in response to temporal and spatial signals. Our findings, and the evolving literature on translational regulation, prompted the possibility that microRNAs (miRs) could play a key role in regulating cellular levels of MKK4 in prostate cancer model systems (5-8).

miRNAs are small noncoding RNAs which have been shown to regulate diverse biological processes in a wide variety of organisms (7). Over the past few years significant progress has been made in delineating the biogenesis of these ~22 nt non-coding RNAs, their involvement in human disease, and tools for their study. Of specific relevance to our data on MKK4 is the role that miRNAs can play in the translational repression of target mRNAs (7). Specifically, miRNAs can repress the production of proteins without necessarily affecting the levels of mRNA, which fits the data we have observed in our prostate cancer cell lines (4). Thus, we hypothesized that miRNAs might control MKK4 translation. Our collaborator Dr. Gorospe had previously identified miRNAs that might associate with the MKK4 3'UTR, including the miR-15, miR-24, miR-25 and miR-141 miRs (9). Thus we began our efforts to test the potential role of these miRs alone or in combination to regulate MKK4 protein levels in various prostate cancer cell lines.

BODY: Although we had promising preliminary data at the time of the previous progress report, we noted some variability in the outcomes of our MKK4 protein depletion studies. In order to address this, control studies were conducted to optimize the experimental technique in our laboratory. Specifically, WI38 human fibroblasts were treated with either control (scrambled) or targeting Pre-miRNAs either singly or in combination [(9); Figure 1]. Cells were transfected with 100nM of each Pre-miR [i.e. Scr, 15b, 24, 25, or 141(Applied Biosystems)] using Lipofectamine 2000 (Invitrogen). The "All miRs" sample was treated with a mixture

Figure 1. Validation of MKK4 depletion by treatment with microRNAs using WI38 human fibroblasts a control. In order to optimize our experimental approach, WI38 cells were treated with microRNAs singly or in combination as (9). After treatment with either scrambled (SCr) or a miR(s) targeting MKK4, protein lysates were prepared from cells and MKK4 levels were assessed by immunoblotting. GAPDH was used as a loading control.
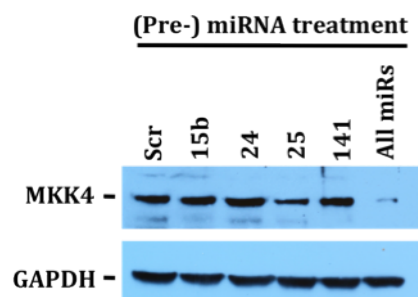
of 15b, 24, 25, and 141 Pre-miRs, each of which at 100 nM (9). Cells remained in regular media containing optimem/lipfectamine complex for 3 days, and then underwent a second transfection. After a total of 6 days of treatment, protein lysates were prepared from the treated cells and MKK4 levels were assessed using immunoblotting (2). As shown in Figure 1, treatment with

the Pre-miR scrambled (Scr) control or Pre-miRs 15b, 24, or 141 did not have a detectable effect on MKK4 protein levels. Treatment with Pre-miR 25 caused a slight decrease in protein levels while the "All miR" treatment depleted MKK4 protein levels to an almost undetectable level.

Since the initiation of this project, additional prostate cancer cell lines have become a focus of work in our laboratory. Thus we re-evaluated the expression of MKK4 protein, mRNA, and miRs in a broader panel of cell lines. These analyses showed a discordance between the relative level of MKK4 protein (Panel A) and MKK4 mRNA (Panel B) in the majority of cell lines. From this survey, we selected the C42-B, 22Rv1, DuPro, LAPC4, LNCaP, and PC3 cell lines for further evaluation. Specifically, the relative level of each of the candidate miRs was determined using real-time PCR. This allowed us to gauge the relative expression of candidate miRs in each cell line and compare it to both the levels of MKK4 protein and RNA.
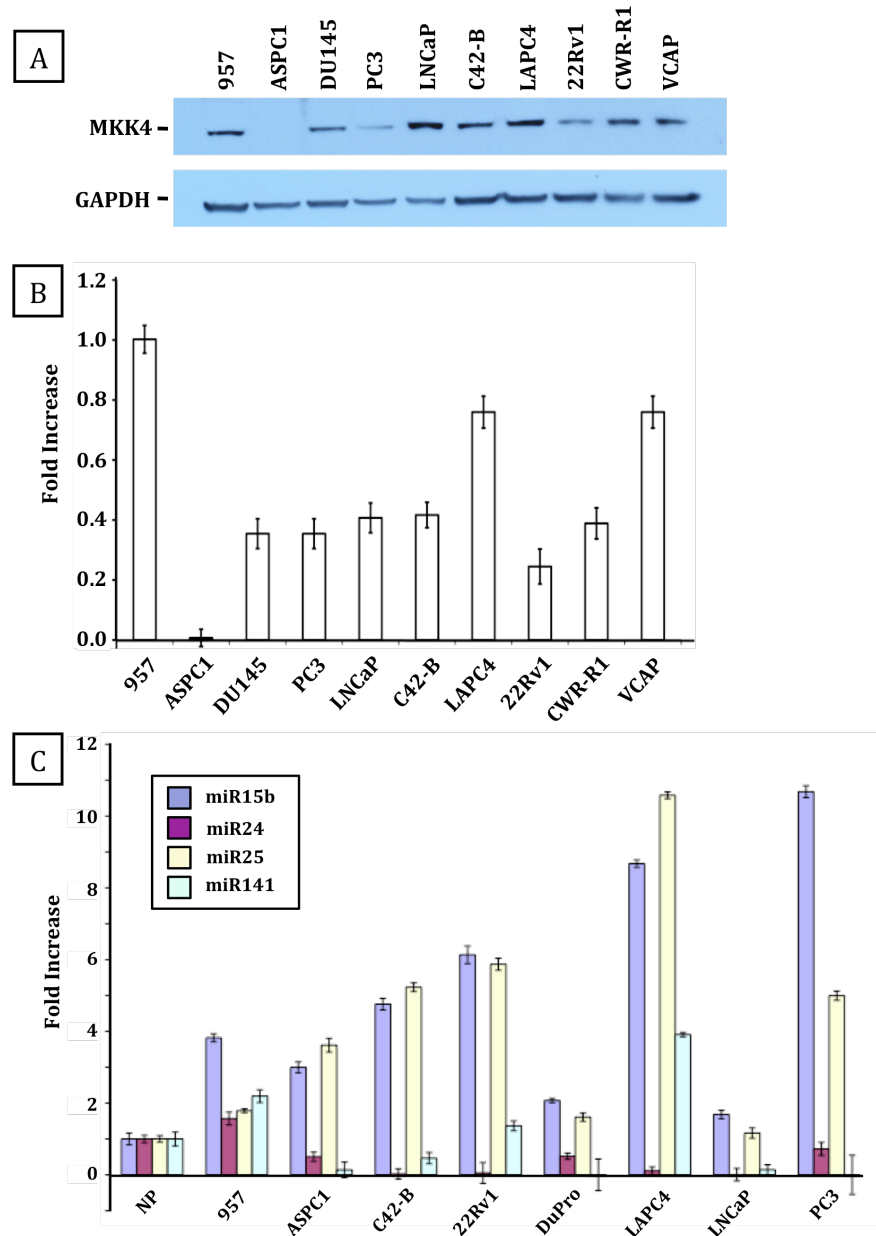

Figure 2. Quantitation of relative levels of MKK4 protein, mRNA and targeting miRs in a panel of prostate cancer cell lines. *Panel A* MKK4 protein was detected using immunoblotting (2). The 957e/h TERT immortalized human prostate epithelial cell line (957) served as the positive control (10); the ASPC1 pancreatic cancer cell line served as the negative control (11). GAPDH was the loading control. *Panel B* The relative level of MKK4 mRNA was determined using real-time PCR. *Panel C* The relative level of each candidate miRs (i.e. 15b, 24, 25, and 141) was determined using real-time PCR. miR levels were normalized to a value of 1.0 in RNA prepared from normal prostate epithelial cells. Error bars represent standard error.

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The preceding data allowed us to prioritize human prostate cancer cell lines for further studies. We were particularly interested in the potential use of the 22Rv1 cell line which was derived from the human prostatic carcinoma xenograft CWR22R (12). 22Rv1 is one of the few available cell lines that recapitulates key aspects of clinical disease. In nude mice, it forms tumors with morphology similar to that of its parental the xenograft and expresses both prostate specific antigen and the androgen receptor. Most importantly, 22Rv1 cells can form bone metastases in severe combined immunodeficient (SCID) mice after intercardiac injection (). As shown in Figure 2, 22Rv1 cells express moderate levels of both MKK4 mRNA and protein, suggesting that it may be feasible to either decrease the level of MKK4 protein (by increasing the cellular level of targeting miRs via Pre-miR treatment) or increase the level of MKK4 protein (by decreasing the cellular level of targeting miRs via Anti-miR treatment). We anticipate that increasing the level of endogenous MKK4 protein will cause decreased metastasis formation and conversely, decreasing the level of endogenous MKK4 protein will cause increased metastasis formation.

To test these possibilities, MKK4 levels were assessed after treatment of 22Rv1 cells with Pre-miRs as indicated in Figure 3, Panel A. Expression of the Scr miR control or miRs 15b, 24, or 141 had no detectable effect on the level of MKK4. In contrast, treatment of cells with a Pre-miR25 alone, or in combination with the other three miRs
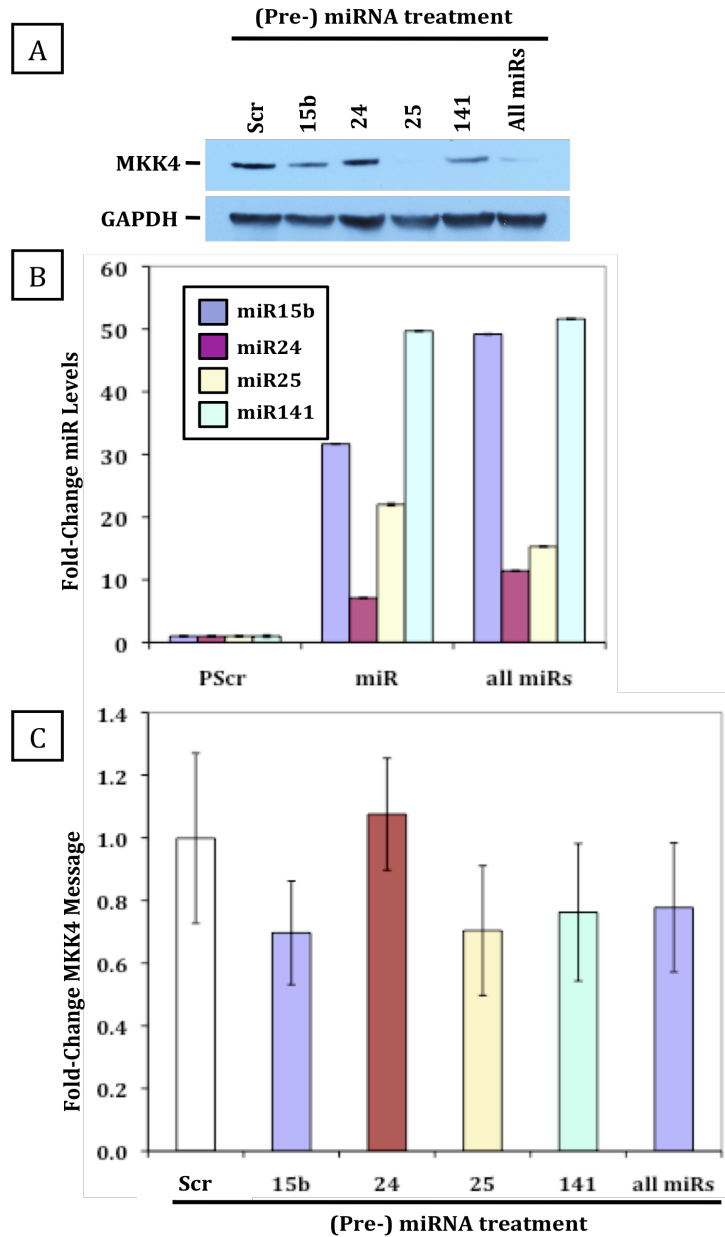

Figure 3. Quantitation of levels of MKK4 protein, specific miRs, and MKK4 message in 22Rv1 cells treated with Pre-miRs singly or in combination. *Panel A* The relative level of MKK4 after treatment with the scrambled (SCR) Pre-miR control (100 nM), individual Pre-miRs (i.e. 15b, 24, 25, or 141) at 100 nM, or a combination of all 4 Pre-miRs (again each at 100nM) was determined using immunoblotting (2). *Panel B* Increased miR expression was confirmed by real-time PCR of each individual miR. Cells were treated with the Scr Pre-miR control (left), individual miRs (middle) or the combination of miRs (right). miR-specific sequences were reverse transcribed from total RNA, and then amplified using real-time PCR. *Panel C* Real-time PCR was used to confirm that Pre-miR treatment did not affect endogenous MKK4 mRNA levels. Error bars represent standard error.

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caused a significant decrease the level of MKK4 protein. To confirm that the effect was due to the increased level of miR in the transfected cells, the relative level of each miR was compare in cells treated with either the Scr control or targeted miR alone (miR) or a combination of all four miRs (all miRs). In all cases, treatment of cells with Pre-miRs caused an increase in the corresponding miRs. As a final control for specificity the level of MKK4 mRNA in treated cells was assessed by real-time PCR. As shown in Figure 3 Panel C, treatment of cells with Pre-miR did not affect the level of MKK4 mRNA. This shows that the reduction in the level of MKK4 in cells treated with Pre-miR 25 is not due to a decrease in the level of MKK4 mRNA. Taken together, the data in Figure 3 demonstrates that we can successfully manipulate MKK4 levels using Pre-miR treatments and this approach should be generalizable to additional cell lines.

As previously stated, a second goal of our work is to increased the level of MKK4 protein by specifically inhibiting miR function via treatment with Anti-miRs which are chemically modified, single stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNA molecules. PC-3 cells were selected for our first effort to increase MKK4 protein through miR inhibition. As shown in Figure 4, Panel A, Anti-miR treatment did not cause a detectable increase in MKK4 levels (as determined by immunoblotting). To confirm that the Anti miR treatment worked as expected, miR and MKK4 mRNA levels were assessed using real-time PCR. As shown in Figure 4, Panel A,
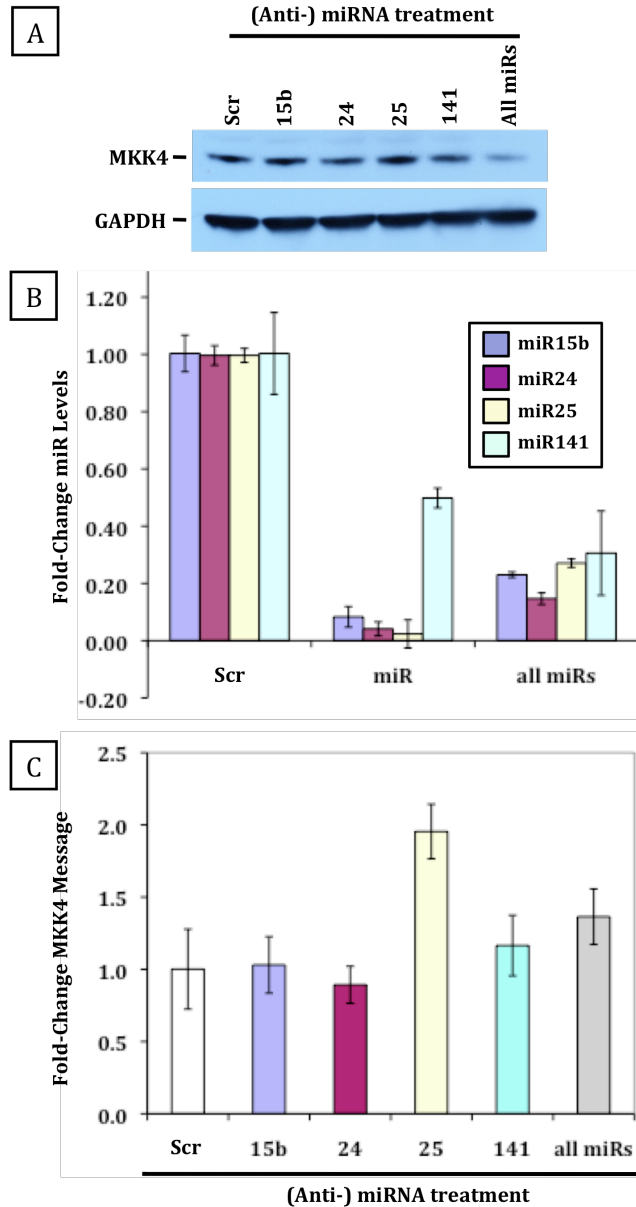

Figure 4. Quantitation of levels of MKK4 protein, specific miRs, and MKK4 message in PC3 cells treated with Anti-miRs singly or in combination. *Panel A* The relative level of MKK4 after treatment with the scrambled (SCR) Anti-miR control (100 nM), individual Anti-miRs (i.e. 15b, 24, 25, or 141) at 100 nM, or a combination of all 4 Anti-miRs (again each at 100nM) was determined using immunoblotting (2). *Panel* B miRNA levels were determined by real-time PCR of each individual miR. Cells were treated with the Scr Anti-miR control (left), individual Anti-miRs (middle) or the combination of Anti-miRs (right). miR-specific sequences were reverse transcribed from total RNA, and then amplified using real-time PCR. The relative level of MKK4 mRNA present in each cell line was determined using real-time PCR. *Panel C* Real-time PCR was used to confirm that Pre-miR treatment did not affect endogenous MKK4 mRNA levels. Error bars represent standard error.

treatment of cells with the Scr Anti-miR control (left) had no significant effect on the miRs available for reverse transcription and subsequent PCR amplification. In contrast treatment with individual Anti-miRs either singly (middle) or in combination (right) decreased the amount of miR available for amplification due to their tight binding to their target sequences. As a final control the level of endogenous MKK4 mRNA was assessed (Figure 4, Panel C). Treatment of cells with Anti-miRs did not cause a significant decreased in the level of MKK4 as compared to cells treated with the Scr Anti-miR control. It is unclear why in this experiment cells treated with Anti-miR 25 had more MKK4 mRNA present. However, this spurious increased did not correspond to any increase in MKK4 protein level, thus, we interpret the finding as the result of sample variability. Taken together these data show that treatment of PC3 cells with Anti-miRs to 15b, 24, 25, and 141 had no significant effect on cellular levels of MKK4.

Using the approaches established in the preceding section we extended our studies into the C4-2B cell line, which can also form bone metastases and was established by the laboratory of Dr. Leland Chung (13). As shown in Figure 5, treatment of C4-2B cells with Pre-miR25 or the combination of 15b, 24, 25, and 141, caused a

Figure 5. Modulating MKK4 protein levels in C4-2B prostate cancer cell lines using Pre-miRs and Anti-miRs. Using approaches described in the preceding Figures, cells were either treated with Pre-miRs (Left) or Anti-miRs (Right) to decrease or increase the level of MKK4 , respectively. The relative level of MKK4 in the treated cells was assessed by immunoblotting. GAPDH was used as a loading control.

significant reduction in the level of MKK4 protein as compared to cells treated with Scr control or Pre-miRs 15b, 24, or 141 alone. In the converse experiment, cells were treated with Anti-miRs as indicated (Figure 5, Right). This preliminary study yielded promising results with treatment with the combination of all Anti-miRs yielding a 2-4-fold increase in the level of MKK4 protein. Additional experiments are currently being conducted to confirm this data.
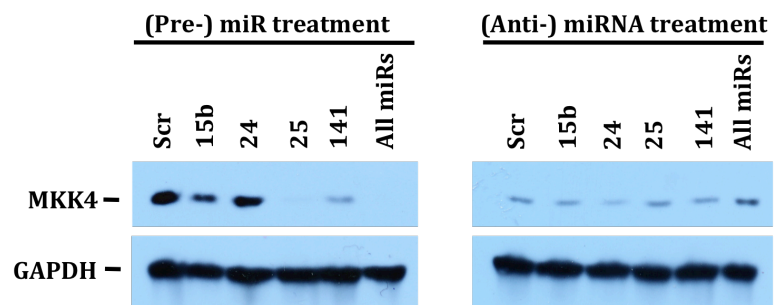
KEY RESEARCH ACCOMPLISHMENTS:

- Optimized conditions for miRNA-mediate depletion of MKK4 levels using treatment of cells with Pre-miRs 15b, 24, 25, and 141, and a combination thereof;

- Determined the relative level of MKK4 protein and mRNA, and the relative levels of four candidate miRs (15b, 24, 25, and 141) in a panel of prostate cancer cell lines;

- Showed that in contrast to WI38 cells, in 22Rv1 and C4-2B cells, treatment with Pre-miR 25 is as effective as treatment with the combination of Pre-miRs 15b, 24, and 141;

- Treatment of PC3 prostate cancer cells with Anti-miRs does not cause an increase in the level of MKK4 protein;

- In agreement with our results from 22Rv1 cells, treatment of C4-2B cells with Pre-miR 25 or the combination of Pre-miRs 15b, 24, 24 and 141 causes a decrease in the level of MKK4 protein; and

- In a preliminary study, treatment of c4-2B cells with a combination of Anti-miRs 15b, 24, 25, and 141 caused a 2-4-fold increase in the level of MKK4 protein.

REPORTABLE OUTCOMES:

None

CONCLUSIONS AND FUTURE DIRECTIONS:

We are pleased that our progress to the end of the second year of this funding we have achieved the key research accomplishments needed to move forward into *in vivo* studies. During the final year of funding we plan to complete:

- The evaluation of Anti- and Pre- miRs to modulate MKK4 levels in both C4-2B and 22Rv1 cells;

- Optimize number and timing of luciferase-tagged cells used for *in vivo* assays; given the *in vivo* properties of these cells we will need to use intracardiac injection of cells followed by bioluminescent imaging;

- Test the effect of modulating MKK4 levels on both the dissemination (distribution) of cells after intracardiac injection and the time course of metastasis formation.

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APPENDICES: NONE

SUPPORTING DATA: Data integrated into the main body of the text.